29 Detection of extra virgin olive oil adulteration

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29.1 Introduction

Virgin olive oil is a valuable plant oil extracted from fresh and healthy olive fruits (*Olea europeae* L.) by mechanical processes (pressing or centrifuging) and without heat, solvents, or any preliminary refining (Ammar *et al.*, 2014a; García & Yousfi, 2006). Therefore, it is practically the only vegetable oil that can be consumed directly in its raw state. Extra virgin olive oil (EVOO) is considered as the best olive oil for its superior organoleptic characteristics (aroma and taste). It has potential health benefits, and remarkable antioxidant properties and chemical composition (Ammar *et al.*, 2014b; Jafari *et al.*, 2009; Méndez & Falqué, 2007). Since EVOO is a premium food product requiring a relatively high price, it is a target for adulteration with less expensive vegetable oils. The most common adulterants found in EVOO are refined olive oil; seed oils, such as sunflower, corn, soybean, and rapeseed oils (Guimet *et al.*, 2005); as well as nut oils, including peanut and hazelnut oils (Blanch *et al.*, 1998). These are used as ingredients in several foods, cooking oils, salad oils, and fried foods. Given the difference in price between EVOO and other edible oils, adding cheaper oils to virgin olive oil has become a common practice for profit. This practice is, however, harmful since consumers buy olive oil for its health benefits (Kafatos & Comas, 1991) and are badly deceived when they receive oil that does not provide them with what they seek. Authenticity covers many aspects, including adulteration, mislabeling, mischaracterization, and misleading origin (Frankel, 2010). Therefore, the detection of edible oil adulteration is crucial in food quality, safety control, and the vegetable oil product trade. Monitoring the authenticity of EVOO is carried out using instrumental techniques that provide data about their qualitative and quantitative composition.

Most of the current work on edible oil adulteration is based on chromatographic analysis, namely gas chromatography (GC), high-performance liquid chromatography (HPLC), and gas or liquid chromatography coupled with mass spectrometry (GC-MS and LC-MS). However, these separating techniques have been complemented with, or substituted by, many other modern fingerprinting techniques, such as Fourier transform near-infrared (FT-NIR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, FT-Raman spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, dielectric spectroscopy (DS), differential scanning calorimetry (DSC), and total synchronous fluorescence spectroscopy (TSyFS). Added to that, a common strategy of these methods is to conduct multivariate statistical analyses of the contents of various components, such as fatty acids and triacylglycerols (TAGs) as well as sterols, and classify samples of different botanical origins using chemometric tools.

Other fingerprinting molecular spectroscopy techniques have been developed and used in recent years. The advantages of the spectroscopy techniques are the negligible sample preparation, the small amounts of organic solvents or reagents used, the noninvasive approach, the relatively easy and quick data acquisition, and the possibility to provide information on a wide range of components in a single experiment maintaining the natural ratio of the substances (Berrueta *et al.*, 2007; Vigli *et al.*, 2003).

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It is in this context that the present chapter examines the effectiveness of both single and coupled (combined) techniques with various analytical instruments to detect the adulteration of EVOO. Here, two main parts are considered: in Section 29.2, the evaluation of authenticity of EVOO followed by various chemical parameters of purities using several sophisticated analytical instruments to detect potential adulterants; and, in Section 29.3, other specific methods are discussed that have been used not only to classify directly oil samples according to botanical origin but also to determine the composition of binary mixtures of EVOO with cheaper oils.

29.2 Parameters suitable for authenticity assessment of EVOO

Olive oil is usually more expensive than other edible oils, which makes it a candidate for adulteration with other cheaper oils. To assess the authenticity of EVOO, it is fundamental to know the technologies applied, the fat modification techniques used, and the chemical composition of the authentic olive oil and that of the potential adulterants. Therefore, different methods have been developed to detect falsification perpetrated.

The central problem for the authenticity assessment of EVOO is to define one or more parameters within the lipid fraction, which allows checking for the identity and purity of the specified olive oil. Ideally, such markers are chemical compounds present in the adulterant oil (soybean, corn, or sunflower oil) and absent in the original one (olive oil). However, very often marker substances are not totally absent in olive oil but present only in concentrations different from that in the adulterated oil. Therefore, the profiles of authentic oils must be compared with the olive oil to be tested.

Chemically, there are two parts of compounds (saponifiable and unsaponifiable matter). In the saponifiable matter, the main constituents of vegetable oils are triacyglycerols (TAGs). The TAGs of EVOO contain mixtures of palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acids and traces of myristic, arachidic, heptadecanoic, and eicosanoic acids. Other constituents include partial acylglycerols (diacylglycerols [DAGs] and monoacylglycerols [MAGs]) and esters of fatty acids with saturated fatty alcohols of linear chain. The unsaponifiable matter, which makes up around 2% of all oils, includes many chemical substances of a very different structure, such as hydrocarbons, tocopherols, pigments, sterols, alcohols, terpenic dialcohols, volatile compounds affecting aroma and flavors, phenolic acid, and flavonoid compounds and proteins.

The detection and determination of the adulteration of EVOO are not simple tasks. Actually, they traditionally require the monitoring of several organic compounds to establish a comparison with typical unadulterated oils so as to identify the change of composition that could be related to adulteration. In this respect, the detection of the characteristics of the chemical components has been proposed as a suitable indication for the presence of other oils in EVOO.

The purity control of EVOO is becoming more stringent, and strict laws are being enforced, especially for avoiding adulteration. The public bodies that are responsible for the prevention of the adulteration of foodstuffs necessitate methods of analysis that could facilitate large-scale *in situ* controls.

29.2.1 Adulteration within fatty acids

The TAGs are composed of three fatty acids attached to a glycerol backbone. Actually, it is the well-balanced fatty acid composition that confers to olive oil its high nutritional values. The composition of the fatty acids of EVOO also depends on several factors, such as soil, climate, processing, harvesting, and changes occurring during storage (Dyer *et al.*, 2008), as well as using peak areas for quantitative analysis without employing an internal standard. The fatty acids of edible oil have always been one of the main issues encountered when dealing with the oil origin and for detection of mixtures, although the wide variation in edible oils from different geographical origins is a limiting factor in the interpretation of data with regard to adulteration (Aparicio & Aparicio-Ruíz, 2000).

The official method for the separation and quantification of cis- and trans-fatty acids from oils involves capillary gas chromatography (GC) analysis, with a high-polar stationary phase (cyanopropyl polysiloxane) and flame ionization detection (FID), after being converted into fatty acid methyl esters (FAMEs) by

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alkali/catalyzed transesterification of vegetable oils. FAMEs are quantified according to their area percentage, obtained by the integration of the peaks. The results were expressed as percentages of individual fatty acids in the lipid fraction (International Olive Council [IOC], 2001a, 2001b).

In order to evaluate the possibility of detecting Chemlali EVOO adulteration with low-cost seed oils, Jabeur *et al.* (2014) prepared various blends of EVOO and soybean, corn, or sunflower oil and analyzed their fatty acids. The adulteration percentages ranged from 1 to 10% to determine a threshold of detection. Fatty acids composition as an indicator of purity suggests that linolenic acid content could be used as a parameter for the detection of EVOO fraud with 5% soybean oil. The adulteration could also be detected by the increase of the trans-fatty acid contents with 3% soybean oil, 2% corn oil, and 4% sunflower oil (Jabeur *et al.*, 2014). It is important to note that most studies based on the compositions of fatty acids have only focused on the detection of adulterations without considering the type of vegetable oil involved.

For determination of the percentage of olive oil in a blend with other types of plant oils (peanut, rice, corn, and grapeseed oils), analysis of FAMEs by GC-FID, followed by chemometric tools (principal component analysis [PCA], target factor analysis [TFA], soft independent models of class analogy [SIMCA], and partial least squares [PLS]), is used. This method leads to the construction of models capable of verifying and recognizing the percentage of olive oil in a binary blend. Good classification models are obtained by adding blends containing 45 and 55% olive oil. In this case, the best results are achieved by applying SIMCA to separate the data sets of binary blends rather than to the overall data set (Monfreda *et al.*, 2014).

Nuclear magnetic resonance (NMR) spectroscopy has become the preeminent technique for determining the structure of organic compounds. The basic information given by NMR is a spectral line characterized by its spectral position (given in parts per million [ppm] relative to a reference frequency) and its intensity. The line intensity is measured as the area under the spectral line and reflects accurately the number of equivalent nuclei in the environment (Mannina *et al.*, 2003). In order to evaluate the composition of the oils and to differentiate between them, ^{1}H and ^{13}C NMR resonance groups are integrated and their percentage from the total signal is estimated.

The ^{1}H and ^{13}C NMR analyses are used to determine the saturated fatty acids, oleic acid, linoleic acid, linolenic acid, and iodine value. The ^{1}H, ^{13}C integrals, and fatty acids concentrations of the 0, 0.5, 1, 5, and 100% olive oil in sunflower oil mixtures were used to make calibration curves (percentage of olive oil in function of the ^{1}H signal/^{13}C signal/fatty acid concentration). These calibration curves were tested by using a sample at 1% (w/w). Popescu *et al.* (2015), using the ^{1}H NMR spectrum, reported 2.77 ppm (—CH═CH—$\underline{CH_2}$—CH═CH– of linoleyl and linolenyl) and 2.03 ppm (—$\underline{CH2}$—CH═CH– of unsaturated fatty acids) are obtained; and from the ^{13}C NMR spectrum, 130.22 (C9 of linoleyl and linolenyl) and 29.42 ppm (CH_2) are obtained in their study. Table 29.1 summarizes the assignment of the principal resonances in the ^{1}H and ^{13}C NMR spectrum of vegetable oil.

NMR is a fast method, faster than most of the techniques used in oil analysis, and it requires only a simple sample preparation. It also enables the recording of more or less all constituents of a complex mixture in a single experiment. This feature can be used for fast screening of a large number of samples and the development of a database for authentic products.

Capote *et al.* (2007) investigated different kinds of adulterants (sunflower, corn, peanut, and coconut oils) in olive oil. However, despite its effective identification, only eight different samples of pure olive oil were considered, which could not eliminate the variability between them (Aguilera *et al.*, 2005; Gargouri *et al.*, 2013). In these, the peak areas were used to quantify without the use of any internal standard.

A semiquantitative method using ^{13}C NMR in the olefinic region (127.5–130 ppm) was reported to detect the presence of plant oils (cottonseed, sunflower seed, soybean, and corn oils) in EVOO, which affected the intensities of 12 peaks and the α/β ratios of oleic acid (1.1) and linoleic acid (1.5) (Mavromoustakos *et al.*, 2000).

Gamazo-Vázquez *et al.* (2003) determined FAMEs after saponification and derivatization by capillary GC coupled to mass spectrometry (GC-MS). This method can be used in bottling plants to efficiently discriminate between pure olive oil and olive oil adulterated by other oils at low percentages. The optimal discriminatory parameter is the ratio between the peak areas of the oleic and linoleic derivatives in the chromatograms obtained using full-scan MS between 35 and 350 amu. This ratio would allow the diagnosis of the contamination of olive oil with sunflower oil at least at the 1% level with >95% certainty in bottling plants.

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Table 29.1 Assignment of the main resonances in the ^{1}H and ^{13}C NMR spectrum of vegetable oil

Chemical shift (ppm) (no.)	^{1}H	Compound
0.87 (1)	$-CH_2-CH_2-CH_2-\underline{CH_3}$	All acids except linolenyl
1.02 (2)	$-CH=CH-CH_2-\underline{CH_3}$	Linolenyl
1.30 (3)	$(\underline{CH_2})n$	All acyl chains
1.62 (4)	$-\underline{CH_2}-CH_2-COOH$	All acyl chains
2.03 (5)	$-\underline{CH_2}-CH=CH-$	All unsaturated fatty acids
2.32 (6)	$-\underline{CH_2}-COOH$	All acyl chains
2.77 (7)	$-CH=CH-\underline{CH_2}-CH=CH-$	Linoleyl and linolenyl
4.22 (8)	$-\underline{CH_2}-OCO-R$	Glycerol (triacylglycerols)
5.26 (9)	$\underline{CH}-OCO-R$	Glycerol (triacylglycerols)
5.37 (10)	$-\underline{CH}=\underline{CH}-$	All unsaturated fatty acids
Chemical shift (ppm)	**^{13}C**	**Compound**
14.07–14.28	C18 (ω1)	All acyl chains
20.56–22.70	C17 (ω2)	All acyl chains
24.49–24.89	C3	All acyl chains
25.56–25.72	C11	Linoleyl and linolenyl
	C14	Linolenyl
27.22–27.38	C8	Oleyl and linoleyl
	C11	Oleyl
29.42	C4–C7	All acyl chains
	C12–C15	Oleyl
	C8–C15	Stearoil
	C8–C13	Palmitoil
31.55–31.94	C16 (ω3)	Linoleyl
34.06–34.20	C2, sn-2	All acyl chains
62.12	CH_2O-, sn-1,3	Glycerol (triacylglycerols)
	CH_2O-, sn-1	Glycerol (1,2-diacylglycerols)
65.07	CH_2O-, sn-1	Glycerol (monoacylglycerols)
68.93	CHO—, sn-2	Glycerol (triacylglycerols)
77.01	CDCl3	Solvent
127.13–127.92	C12	Linoleyl
	C10, C15	Linolenyl
128.10–28.47	C10	Linoleyl
	C12, C13	Linolenyl
129.49–129.70	C9, C10	Oleyl
130.02–130.45	C9	Linoleyl and linolenyl
	C13	Linoleyl
172.81	C1, sn-2	Triacylglycerols
173.23	C1, sn-1,3	Triacylglycerols

Ozen *et al.* (2003) successfully classified adulterated and pure oil and the detection limit for adulteration. FTIR and common chemometric techniques (including discriminant analysis [DA], Mahalanobis distances, and Cooman plots) were used to classify various types of dietary supplement oils (DSOs) and cheaper edible oils. These analyses indicated that the developed FTIR method and chemometric analysis are very useful for quantifying the adulterant oil added to the DSO at the 2–20% (v/v) level.

29.2.2 Triacylglycerols

TAGs generally account for 95–98% (w/w) of vegetable oils and show a characteristic distribution. As a consequence, the addition of other edible vegetable oils to olive oils modifies TAG distribution, and because of that, they are considered to be good fingerprints for adulteration detection purposes (Aparicio & Aparicio-Ruíz, 2000).

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The reverse-phase high-performance liquid chromatography (RP-HPLC) quantitative analysis of TAGs is considered as an effective method for the detection of EVOO adulteration (De la Mata-Espinosa *et al.*, 2011). In this case, the stationary phase usually consists of a nonpolar octadecylsilane (C_{18}) bonded phase, while the mobile phase is a polar solvent (acetonitrile/acetone [50:50, v/v]). The refractometer detector is most appropriate for quantitative analysis carried out using thermostated cells and isocratic elution. The advantage of using a TAG profile includes the distribution of fatty acids between the different stereospecific positions on the glycerol molecule. TAGs are separated according to the equivalent carbon number (ECN) and the positions of double bond(s). Until recently, the most prominent methods to detect the adulteration of EVOO with other vegetable oils was the trilinolein (LLL) content and the difference between the theoretical value of TAGs with an equivalent carbon number of 42 ($ECN42_{theoretical}$). An appropriate software is used to compute the ΔECN42 based on data of the fatty acids composition and analytical triacylglycerol results ($ECN42_{HPLC}$) (IOC, 2010; García-González *et al.*, 2007). Better results were obtained by Jabeur *et al.* (2014) using ΔECN42, which proved to be effective in the Chemlali EVOO adulteration at levels as low as: 1% sunflower oil, 3% soybean oil, and 3% corn oil. TAG compositions of EVOO and the adulterated EVOO oil mixed with 10% (w/w) are depicted in Figure 29.1.

High-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (HPLC-APCI-MS) was used to determine the adulteration of EVOO with 10–50% hazelnut oil based on TAG composition and non-TAG components (Parcerisa *et al.*, 2000). Discriminant analysis (DA) showed that hazelnut oil and mixtures with olive oil were clearly separated according to their TAG composition.

Lerma-García *et al.* (2011) developed a new method for the determination of TAGs in vegetable oils from different botanical origins by using HPLC with ultraviolet-visible (UV-Vis) detection. Using a core–shell particle packed column (C_{18}, 2.6 μm), isocratic elution with acetonitrile/*n*-pentanol at 10 °C and within a total analysis time of 15 min was achieved. Using mass spectrometric detection, a total of peaks, which were common to the oils of six different botanical origins (corn, EVOO, grapeseed, hazelnut, peanut, and soybean oils), were identified. These peaks were used to construct linear discriminant analysis (LDA) models

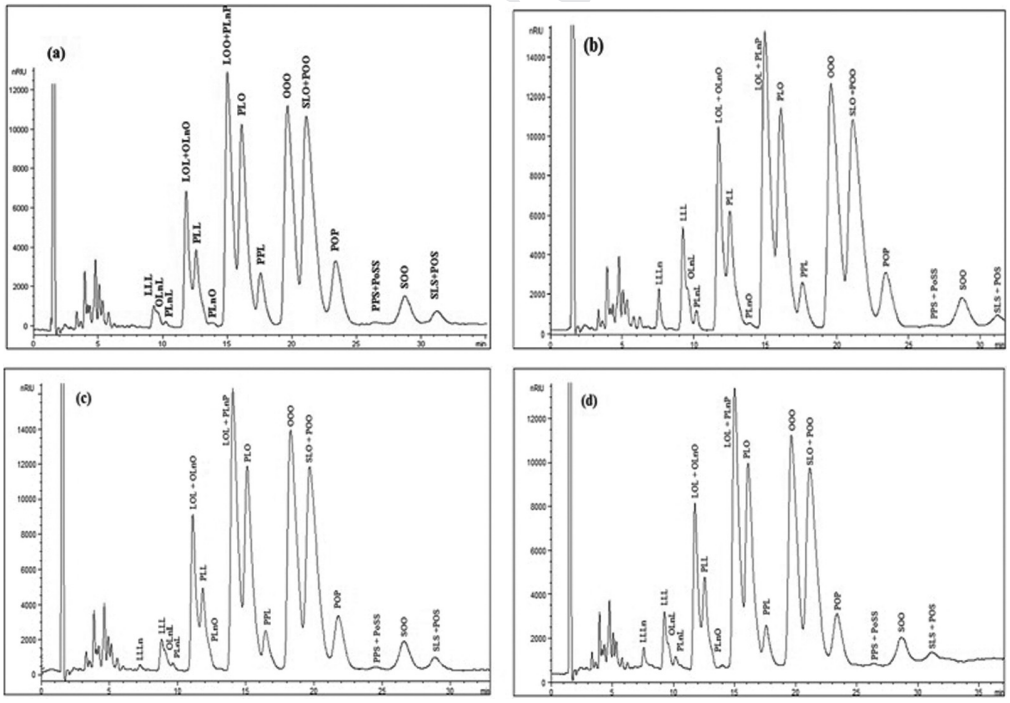

Figure 29.1 Chromatograms showing the (a) TAG profiles of Chemlali EVOO; and binary mixtures containing 90% EVOO and 10% of either (b) soybean, (c) sunflower, or (d) corn oils using HPLC-RID.

for botanical origin prediction. Afterward, and in order to evaluate the possibility of detection of EVOO adulteration with low-cost oils, binary mixtures containing 90% EVOO with 10% of either corn, grapeseed, hazelnut, peanut, or soybean oils were prepared. When EVOO was adulterated with grapeseed oil, the relative areas of OLL (oleic-linoleic-linoleic), LLP (linoleic-linoleic-palmitic), and particularly LLL largely increased. Similarly, the presence of hazelnut oil was evidenced by an increase of the areas of LLL and OLL. The adulteration with corn oil resulted in a large increase in the areas of LLL, OLL, and LLP. When peanut oil was present, the areas of LLL, OLL, LLP, and LLBe increased largely. Finally, the adulteration with soybean oil produced a large increase in the areas of LLLn (linoleic-linoleic-linolenic), LLL, OLLn (oleic-linoleic-linolenic), OLL, and LLP. Therefore, adulteration of EVOO with small percentages of other oils was clearly proven in all cases, although with a moderate sensitivity for hazelnut oil (Lerma-García *et al.*, 2011).

Cunha and Oliveira (2006) used HPLC–evaporative light scattering detection (ELCD) to determine TAG composition in oils. The chromatographic separation was achieved using a Kromasil 100 C_{18} column (at 25 °C) and gradient elution with acetone and acetonitrile. After the methodology implementation and validation, it was applied to the study of the TAG profiles of eight plant oils (sunfiower, corn, peanut, soybean, hazelnut, walnut, sesame, and olive oil). A categorical principal component analysis (CATPCA) was performed to simplify the data from TAG profiles of vegetable oils, and to easily distinguish vegetable oils except hazelnut oil. These oils can be differentiated from the others by their high levels of triolein (OOO). Sunflower and walnut oils are discriminated from the other oils, mainly with LLL and palmitodilinolein (PLL) parameters, which also permit the differentiation between both of them. On the other hand, the other plant oils (peanut, corn, sesame, and soybean) have different profiles mainly pertaining to the contents of PLO (palmitic-linoleic-oleic), SPO (stearic-palmitic-oleic), and POP (palmitic-oleic-palmitic). Furthermore, the determination of TAG composition may be an important parameter for detecting the adulteration of such products during purity control.

The optimization and application of methods of triacylglycerol evaluation for characterization of olive oil adulteration by soybean oil with HPLC-APCI-MS/MS (tandem MS) have been developed by Fasciotti and Pereira-Netto (2010). HPLC separation was also carried out using an octadecylsilica LiChrospher column (250 mm × 3 mm; 5 μm) and a gradient composed of acetonitrile and 2-propanol. APCI-MS run in positive mode and an ion trap mass analyzer were applied in the study of olive and soybean oils and their mixtures. Multiple reaction monitoring (MRM) employing the transition of protonated TAG molecules ($[M+H]^+$) to the protonated diacylglycerol fragments ($[M+H{-}R]^+$) improved the selectivity of TAG detection and was used for quantitative analysis. The quantitative studies were based on the estimates of mixtures of soybean oil and olive oil proportions by comparison of TAG areas found in the mixtures of both oils. Good agreement with expected or labeled values was found for a commercial blend containing 15% (w/w) olive oil in soybean oil and in a 1:1 mixture of both oils, showing the potential of this method in characterizing oil mixtures and estimating oil proportions. Olive oils of different origins were also evaluated by mass spectral data obtained after direct injection of oil solutions and PCA.

A reliable procedure for identification and quantification of adulteration of olive oils in terms of blending with other vegetable oils (sunflower, corn, seeds, sesame, and soybean) was developed by Ruiz-Samblás *et al.* (2012). The high-temperature GC method proposed, chemometric class-modeling techniques such as SIMCA, and quantification techniques PLS and genetic algorithms (GA-PLS) with feature selection appear to be appropriate tools to verify the percentage of olive oil in blends with vegetable oils; and they could become an important instrument to verify the labeling compliance and for quality control in the detection of adulteration. Indeed, reliability of the proposed qualification model is very high as the kind of vegetable oils used for blending was correctly identified for all samples. Moreover, reliable quantification models were built for each of the different blend kinds. Lastly, for the possibility of quantifying the purity of oil samples regardless of the adulterating vegetable oil, promising results were obtained by applying PLS (on the entire chromatogram or with GA variable selection) to the whole data set without preliminary classification of the oils.

29.2.3 Sterols

Sterols are part of the unsaponifiable matter and are found in almost all fats and oils, including EVOO. They are also characteristics of the genuineness of vegetable oils (Gargouri *et al.*, 2015). Indeed, several

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phytosterols are present in virgin olive oil, mainly β-sitosterol, which is the dominant member of the total sterol fraction (Kiritsakis, 1998). In general, sterols are useful markers and fingerprinting components for assessing authenticity of oils. Considering that β-sitosterol is the most abundant in a majority of oils, its value has only limited use for the authenticity assessment and differentiation of vegetable oils. However, it has been shown to be useful for tracing vegetable oils in the fats of animal origin, as the latter contains cholesterol as the primary sterol.

While EVOO has elevated levels of β-sitosterol and Δ5-avenasterol, safflower and sunflower oils contain significant levels of Δ7-stigmastenol, whereas soybean and corn oils have high levels of campesterol and stigmasterol, yet brassicasterol is mainly present in rapeseed and mustard seed oils. These apparent differences make them most suitable for determining the botanical origin of oils and, hence, for detecting the adulteration of olive oil with other cheaper or lower quality vegetable oils (Bohačenko *et al.*, 2001; Galeano-Diaz *et al.*, 2005). Thus, the composition of the sterol fraction of olive oil is a very useful parameter for detecting the adulteration or to check authenticity, since it can be considered as a fingerprint.

Chromatographic methods are currently most widely used for qualitative and quantitative analyses of this extensive series of compounds clustered in 4-demethylsterols or simply sterols. Indeed, gas chromatography of unsaponifiable matter is the most prevalent technique.

An official method for the isolation of total sterols from EVOO and EVOO adulteration implies the saponification of the oil, extraction of the unsaponifiable matter with diethyl ether, and washing of the extract with water. The extract is fractionated by thin-layer chromatography (TLC) on silica gel. Injection is usually performed after derivatization with a silating reactant. Capillary columns give the best performance since they can resolve the sterols almost completely (IOC, 2001c).

A recent study by Jabeur *et al.* (2014) reported that the sterols profile is almost decisive in clarifying the adulteration of olive oils with cheaper oils; 1% sunflower oil could be detected by the increase of Δ7-stigmastenol, and 4% corn oil by the increase of campesterol. Moreover, they have confirmed that linear discriminant analysis (LDA) could represent a powerful tool for faster and cheaper evaluation of EVOO adulteration (Figure 29.2).

Al-Ismail *et al.* (2010) were able to detect the adulteration of virgin olive oil with some refined vegetable oils by direct sterol analysis using gas–liquid chromatography (GLC) equipped with a polar column and high thermal stability. This method is based on the determination of the sum of campesterol and stigmasterol percentages. Mixtures of corn, soybean, sunflower, and cottonseed oils in olive oil at levels of 5, 10, and

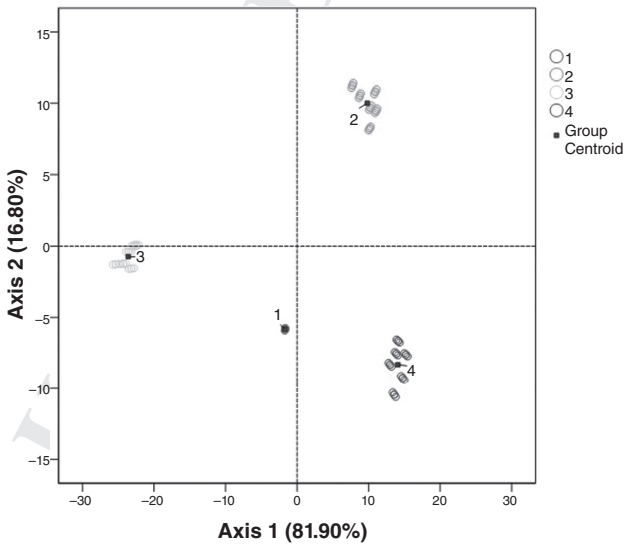

Figure 29.2 LDA score plot of pure EVOO and adulterated EVOO based on all the analyses performed with four determinations. (1) EVOO: extra virgin olive oil; (2) EVOO + (1–10%) of soybean oil; (3) EVOO + (1–10%) of corn oil; and (4) EVOO + (1–10%) of sunflower oil.

20% were studied. An olive oil authenticity factor based on the summation of campesterol and stigmasterol percentages was established as an indicator of olive oil adulteration with vegetable oils. The results indicate the possibility to detect the addition of these vegetable oils in olive oil at less than 5%.

For identification of brassicasterol in olive oil, GC–electron ionization–MS (GC-EI-MS) of their sterols has been used. Brumley *et al.* (1985) detected the presence of brassicasterol in EVOO by the addition of rapeseed or canola oil, taking into account that the content of brassicasterol is ≤0.1% for olive oil, while it is ≥12 and 5% in rapeseed and canola oils, respectively.

Grob *et al.* (1994a) detected the adulteration of virgin olive oil with plant oils (rapeseed, soybean, sunflower, and grapeseed) by direct analysis of the sterols using on-line coupled LC-GC-FID. The addition of approximately 2% rapeseed oil was detected by determining the content of brassicasterol. The addition of 10% soybean oil was detected by determining the concentration of campesterol and stigmasterol. Besides, the contamination of olive oil by sunflower oil higher than 5% could be detected by the increase of campesterol, stigmasterol, and especially Δ7-stigmastenol. Adulteration with 10% or more of grapeseed oil would increase the concentration of campesterol and stigmasterol.

A strategy to avoid the detection of the adulteration of olive oil with sunflower oil is the elimination of the Δ7-sterol fraction by refining under extreme conditions (oils treated strongly with bleaching earth). However, in the course of this process, the Δ5-sterols are dehydrated to sterenes and Δ7-sterols undergo isomerization to Δ8 (14) and Δ14-sterols. The determination of Δ8 (14) and Δ14-sterols has been proposed for detecting desterolized high-oleic sunflower oil in olive oil, since these sterols arise from the isomerization of Δ7-stigmastenol. After elimination of a majority of β-sitosterol by means of TLC on silica gel plates, these reaction products can be detected by GC equipped with a polar column (70% phenylmethylsilicone) (Mariani & Bellan, 2011).

29.2.4 Stigmasta-3,5-diene

In comparison with EVOO, refined vegetable oils are characterized by stigmasta-3,5-diene; the origin of this hydrocarbon, which has been studied by Cert *et al.* (1994), is the dehydration of β-sitosterol during the refining of edible oils and fats (see the mechanism of sterol dehydration in Figure 29.3). This hydrocarbon is very useful in detecting the presence of refined oil in crude oils. Many vegetable oils are refined by different steps, including bleaching and deodorization, by treatment with acid bleaching earth and steaming at high temperatures. These processes dehydrate the sterols present in the oil to a series of steroidal hydrocarbons or sterenes (Cert *et al.*, 1994). The major plant sterol is β-sitosterol from principally 24-ethylcholesta-3,5-diene

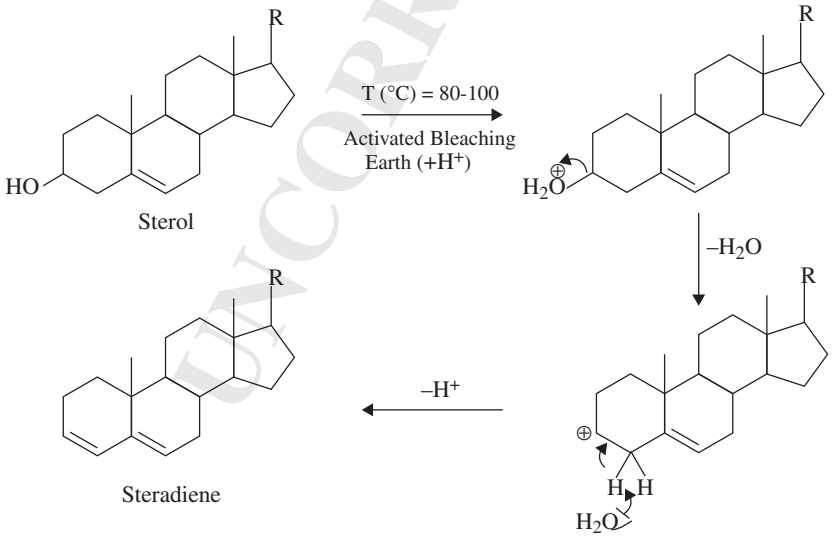

Figure 29.3 Mechanism of sterols dehydration.

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(stigmasta-3,5-diene) and lesser quantities of its positional isomers. Other sterols form similar compounds: for example, 24-methylcholesta-3,5-diene (campesta-3,5-diene) is from campesterol, and 24-ethylcholesta-3,5,22-triene (stigmasta-3,5,22-triene) is formed from stigmasterol.

Currently, adulteration with refined vegetable oils is a major issue in the EVOO market. The stigmastadiene test is the most effective means of detecting this type of adulteration.

The official methods for determination of stigmastadiene in virgin olive oil have been adopted by the IOC (2001d) with an application limit between 0.01 and 4 mg kg^{-1}. The isolation of steroidal hydrocarbons in virgin olive oil is usually performed using low-pressure column chromatography on the silica gel of the unsaponifiable matter of 20 g of oil, using *n*-hexane as eluent. For determination of stigmastadiene in virgin olive oil, the first fraction eluted from the column is discarded, and the second one is analyzed by GC on a fused-silica capillary column coated with 5% phenylmethylpolysiloxane and equipped with a FID detector. Stigmastadiene is quantified by the comparison of the combined peak area with that of a single point addition of an internal standard of cholestadiene (IOC, 2001d). Steroidal hydrocarbons in refined oils are found in higher concentrations than in crude oils, and the saponification step can be avoided. The oil dissolved in hexane is directly fractioned on a silica gel column, and the corresponding fraction analyzed (IOC, 2001e).

In this context, Crews *et al.* (2014) described a rapid GC-MS method for the determination of stigmastadiene, which is faster and more sensitive than the current official procedure, based on GC-FID. This method does not require a saponification step for cold-pressed oils, but rather uses a stigmastadiene standard for quantification. It has a low limit of quantification (0.015 mg kg^{-1}) and gives excellent confirmation of peak identity at the current regulatory limit of 0.5 mg kg^{-1}. The availability of selected ion monitoring allows the detection of other sterenes in vegetable oils, which can help in identifying the source of added plant oils. By simple inclusion of additional fragment ions, other sterenes can be monitored. For example, monitoring of the response at m/z 394 and measuring against the available standard allow the quantification of stigmasta-3,5,22-triene, and monitoring of the response at m/z 381 allows the detection of campesta-3,5,22-triene derived from refined rapeseed oil.

The 3,5-steradienes compounds are the main dehydration products of Δ5-sterols, but other isomers are also formed along with the degradation products of Δ7-sterols, methylsterols, and triterpenic alcohols. In order to identify these minor compounds, new isolation procedures have been employed by Grob *et al.* (1994b), using on-line HPLC-GC-MS on silica gel columns to identify 3,5-, 2,4-, and 2,5-steradienes; the 3,5-cyclo-6-enes; and 2,4,6-trienes. The first column separates the sterenes (except squalene) from the triacylglycerols and other polar compounds, and the second one separates different fractions identified with UV detection: stereradienes at 235 nm and steradiene at 309 nm.

Jabeur *et al.* (2015b) recently showed that the profile of sterenes is almost decisive in clarifying the contamination of EVOO with some cheaper refined plant oils. The use of stigmasta-3,5-diene proved to be more effective in detecting even low levels of adulteration of Chemlali EVOO with most of the refined vegetable oils under study. The determination of stigmasta-3,5-diene can be used as a parameter for the detection of EVOO fraud with each studied refined oil: 2% olive (0.081 ppm > 0.05 ppm), 0.4% pomace olive (0.062 ppm > 0.05 ppm), 1% palm (0.063 ppm > 0.05 ppm), 0.2% soybean (0.069 ppm > 0.05 ppm), 0.5% sunflower (0.062 ppm > 0.05 ppm), and 0.1% corn (0.065 ppm > 0.05 ppm) oils.

29.2.5 Fatty acid alkyl esters

Fatty acid alkyl esters (FAAEs), mainly fatty acid ethyl esters (FAEEs) and FAMEs, represent the family of natural neutral lipids present in olive oils and formed by the esterification of free fatty acids (FFAs) with low molecular alcohols, such as methanol and ethanol. They can easily occur in an acid medium and are catalyzed by the presence of certain enzymes.

Blends between EVOO and mild thermal deodorized oil do not produce the easily detectable modifications of the chemical composition. Indeed, the parameters usually checked as quality indicators, such as TAG composition, sterols, and newly formed steroid hydrocarbons, are not appreciably altered by blending (Saba *et al.*, 2005). The most reliable technique seems to be the determination of FAAEs and FAMEs, which are present in the waxy fraction of olive oils (Biedermann *et al.*, 2008). In good-quality EVOOs, FAMEs and FAEEs are present in very small amounts, yet they are present in higher amounts in lampante virgin olive oils (Mariani & Bellan, 2011) and in the second olive-processing oil (the so-called *repaso*) (Cerretani *et al.*, 2011).

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Saba *et al.* (2005) identified 9(*E*), 11(*E*) octadecadienoate (C18:2) in the FAMEs by means of GC coupled to acetonitrile CI-MS and CI-MS/MS of some extra virgin and deodorized olive oils. From the obtained results, the conjugated linoleic acid methyl ester formation is related to the heating temperature and time in olive oil samples; therefore, it may represent a good marker for the detection of deodorized oil addition to EVOO at a very low level.

An analytical procedure for quantitative determination of FAAEs together with squalene in vegetable oils has been developed (Pérez-Camino *et al.*, 2002). The fraction containing these compounds was isolated from the oil by solid phase extraction (SPE) on silica gel cartridges and then quantitatively analyzed by GC. This method was applied to extra and lampante virgin olive oil categories as well as to oils obtained from olive pomace by second centrifugation and solvent extraction. EVOO oils contained low amounts of FAMEs and FAEEs, while oils obtained from altered olive or olive pomace showed high concentrations of FAAEs, mainly ethyl esters. The correlation between oil acidity and ethyl esters concentration was poor.

The influence of soft deodorization on the composition of FAMEs and FAEEs in olive oils was investigated by Pérez-Camino *et al.* (2008) using GC. FAAEs can be considered as good markers of low-quality olive oil subjected to soft deodorization (Jabeur *et al.*, 2015a). From all data analyses on the studied oils, it can be confirmed that the virgin olive oil contains less than 70 mg/kg (mean value, 24.3) FAAEs and a FAEE–FAME ratio lower than 2. Other oils that comply with the analytical requirements actually established for EVOOs and having higher FAAEs and FAEE–FAME ratios are suspected of being subjected to soft deodorization. The total amount of methyl and ethyl esters of fatty acids (FAAEs) and the ratio between ethyl and methyl esters (i.e., the ratio of FAEEs to FAMEs) of the adulterated EVOO mixed with 1–50% (w/w) mild deodorized olive oil are summarized in Table 29.2.

The fast Fourier transform mid-infrared (FT-MIR) spectroscopy combined with PLS methodology has been used by Valli *et al.* (2013) for predicting the level of low-quality virgin olive oil adulteration in EVOO. Results were statistically similar to the official procedure (IOC, 2009) in terms of analytical performance for the total amount of FAMEs and FAEEs and the ratio between ethyl and methyl esters in EVOO; there was a good agreement between the predicted and actual values on calibration data sets was 0.98 and 0.83, respectively, and the limit of quantification was low enough (29.3 mg kg^{-1}), the actual limits for Σ (FAMEs + FAEEs).

29.2.6 Adulteration with copper–chlorophyll

Carotenes present in olive oil are responsible for its yellow color, while chlorophylls are responsible for the greenish color. The presence of these pigments in olive oil not only determines its color but also plays an important role in its oxidative stability due to their antioxidant activity in the dark and pro-oxidant activity in the light (Kiritsakis, 1998; Ouesleti *et al.*, 2009).

Some oils marked as virgin olive oils have been found to contain synthetic pigment Cu–chlorophyll (E141) or other green pigments, added illegally. An analytical method for identification and quantification of Cu–chlorophyll adulteration in edible oils has been developed by Fang *et al.* (2015). High-resolution MS with a high-performance liquid-quadripole (HPLC-Q)-Orbitrap system and HPLC coupled with photodiode-array detector analyses are applied to a survey of E141 pigment in commercial plant oils, including olive pomace oil, EVOO, olive oil, grapeseed oil, and blended oils. From all data analyses on the studied oils, it can be confirmed that the presence of Cu–chlorophyll derivatives is indicative of fraudulent adulteration of oils.

29.3 Direct authenticity assessment of EVOO

To prevent oil adulteration, antifraud controls require that specific tests be performed to assess the quality of EVOOs, which usually rely on highly sophisticated and expensive methods. The chemical methods officially employed for the control of authenticity of virgin olive oil, such as GC and HPLC, are expensive and time-consuming and require skilled operators.

New and complementary analytical techniques devoid of such troubles could act as supporting tools for currently used methods. Among them, calorimetric techniques seem to be very promising, and the application of DSC to make evident the adulteration of EVOO has been reported by Chiavaro *et al.* (2008). The

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Table 29.2 Content of fatty acid ethyl esters in the mixtures of extra virgin olive oil with deodorized olive oil (DO)

	Grams of DO added to EVOO in 100 g of oil mixture									
	0	**1**	**5**	**10**	**15**	**20**	**30**	**40**	**50**	**100**
FAMEs (ppm)	6.80±0.61	8.20±0.34*	16.20±0.58***	21.00±0.36***	30.30±0.27***	35.20±0.42***	47.80±0.55***	53.70±0.77***	66.40±0.51***	108.30±1.23***
FAEEs (ppm)	5.00±0.21	7.80±0.50***	18.00±0.37***	26.40±0.67***	41.70±1.09***	50.00±0.31***	74.30±0.69***	87.60±0.41***	121.20±1.66***	271.70±0.86***
Total FAAEs (ppm)	11.80±0.82	16.00±0.84**	34.20±0.95***	47.40±1.03***	72.00±1.36***	85.20±0.73***	122.10±1.24***	141.30±1.18***	187.60±2.17***	380.00±2.09***
FAEEs/FAMEs	0.73	0.95	1.11	1.25	1.37	1.42	1.55	1.63	1.82	2.50
Classification	EVOO	EVOO	EVOO	EVOO	EVOO	EVOO	VOO	VOO	VOO	DO

Note: EVOO = extra virgin olive oil; FAEEs = fatty acid ethyl esters; FAMEs = fatty acid methyl esters; VOO = virgin olive oil. Each value represents the mean of three determinations ($n = 3$) ± standard deviation. Significant differences between the EVOO and the mixtures of EVOO with deodorized olive oil (DO) groups.*
$p < 0.05$;** $p < 0.01$; *** $p < 0.001$.

potential application of DSC to verify the adulteration of EVOO with refined hazelnut oil was evaluated. EVOO and hazelnut oil were characterized by significantly different cooling and heating DSC thermal profiles. The addition of hazelnut oil significantly enhanced crystallization enthalpy (at hazelnut oil ≥20%) and shifted the transition toward lower temperatures (at hazelnut oil ≥5%). Finally, both cooling and heating DSC thermograms undergo significant changes as a result of the addition of hazelnut oil to EVOO, and they may be a tool for the detection of adulteration of EVOO with refined hazelnut oil.

Two methods to quantify the adulteration of EVOO based on physical characteristics of adulterated samples have been described by Torrecilla *et al.* (2011). Firstly, the adulterant agent concentration is determined using the density and/or refractive indices (RIs) of the adulterated samples of EVOO with sunflower or corn oils by suitable linear correlations between density and/or RI. Finally, the models based on the combination of DSC equipment and a chaotic parameter (lag-k autocorrelation coefficients [LCCs]) are defined here to quantify the adulterations of EVOO with refined, refined olive pomace, sunflower, or corn oils. In both studied models, the adulterant agent concentrations were less than 14% (w/w). The former is adequate to calculate the concentration of the adulterant with a correlation coefficient (R^2) higher than 0.927 and a mean square error (MSE) less than 8.9%.

Microwave dielectric spectroscopy appears as a promising solution, and it has potential as a tool for adulteration detection and for monitoring vegetable oils. The dielectric spectroscopy has become an effective method for qualitative characterization and other intrinsic physical characteristics of foodstuff materials (Miura *et al.*, 2003; Venkatesh & Raghavan, 2004). To this end, the Cole-Cole dielectric parameters (and, in particular, relaxation frequency) of different vegetable oils were evaluated through an innovative automatable procedure. Successively, typical cases of adulteration conditions were considered by mixing olive oil and sunflower oil in different percentages. In this case, the relaxation frequency appears to be the Cole-Cole parameter that indicates the presence of adulterants (Cataldo *et al.*, 2010).

The correct discrimination of EVOO samples were obtained by HPLC-MS with direct injection and positive APCI detection without chemical derivatization and purification by using stepwise discriminant function analysis (SDFA) to select the variables and LDA (Nagy *et al.*, 2005). The correct classification and 99% prediction rate were obtained with samples from three Italian olive cultivars. The authors claim qualitative detection of adulteration to be above 91 and 88% identification of the type of adulterant (sunflower, corn, peanut, and coconut oils).

Direct infusion ESI-MS was used to differentiate qualitatively unrefined olive oil from vegetable oils, to detect the aging and adulteration of vegetable oils by analyzing the polar components extracted with methanol–water (1:1) from different oils and mixtures (Catharino *et al.*, 2005). PCA was used to distinguish unique major diagnostic ions of olive oil from those of other vegetable oils (soybean, corn, canola, sunflower, and cottonseed). The corresponding ESI-MS fingerprints in the negative mode also differentiate olive oil from the other refined vegetable oils and oxidized soybean oil, showing additional ions than those in fresh oil.

Mid-IR and FTIR spectroscopy combined with chemometrics (PLS and PCA models) were used to detect and quantify the adulteration of EVOO with edible oils (Gurdeniz & Ozen, 2009). The model, based on PLS analysis developed to detect adulteration, was limited to 10%. In another study, FTIR was used to classify oil samples according to botanical origin and determine the composition of the binary mixtures of EVOO with cheaper oils (sunflower, corn, soybean, and hazelnut oils) (Lerma-García *et al.*, 2010). Absorbance peak areas were normalized within the FTIR spectra as predictors of botanical origin by LDA. Multiple linear regression (MLR) models were used to determine binary mixtures as low as 5% of EVOO with other vegetable oils.

High-power gradient NMR diffusion coefficients (D) were determined to detect the adulteration of EVOO for the rapid screening of adulteration of olive oils with cheaper vegetable oils (Šmejkalová & Piccolo, 2010). Changes in D values could be detected with the adulteration of 10% for sunflower and soybean oils and 30% for hazelnut and peanut oils.

Dielectric spectroscopy (DS) was adopted for quantitative determination of the levels of adulterant in olive oil (Toyoda, 2003). As a simple, rapid, and nondestructive measuring technique, DS provides information about the dielectric response of materials to electromagnetic fields. The dielectric spectra of a binary mixture of olive oil spiked with other oils increased linearly with the increase in the concentration of soybean, corn, canola, sesame, and perilla oils from 0 to 100% (w/w). The dielectric properties of the binary mixture of oils were investigated in the frequency range of 101 Hz–1 MHz. A PLS model was developed and used to verify

the concentrations of the adulterant. Furthermore, PCA was used to classify olive oil samples as distinct from other adulterants based on their dielectric spectra (Lizhi *et al.*, 2010).

Maggio *et al.* (2010) developed a multistage strategy combining FTIR with PLS as a multivariate method for monitoring the purity of EVOO and performing qualitative and quantitative determinations of adulterants (canola, hazelnut, pomace, and high-linoleic/high-oleic sunflower) in commercial samples. This general operating procedure represents an improvement toward adulterant assessment in EVOO, using the prediction of adulterant ratio and the spectral residues to determine sample composition. The method developed was suitable for determination of modeled adulterants, but it may also reveal an adulteration.

Downey *et al.* (2002) used visible and near-infrared transflectance spectroscopy to discriminate between authentic EVOOs and the same oils adulterated with the addition of sunflower oil, and to quantify the level of sunflower oil adulterant present. A number of multivariate mathematical approaches were investigated to detect and quantify the sunflower oil adulterant. These include hierarchical cluster analysis, SIMCA, and PLS regression. SIMCA can successfully discriminate between authentic EVOO and the same oils adulterated with sunflower oil at levels as low as 1% (w/w). The greatest classification accuracy was achieved using the first derivative of spectral data in the wavelength range of 1100–2498 nm. Using a confidence level of 1%, a 100% correct classification was achieved in both calibration and prediction sample sets.

Papadopoulos *et al.* (2002) used chemiluminescence (CL) for the detection of the adulteration of the more expensive EVOO with cheaper plant oils (corn and sunflower). The energy is produced by the oxidation of polyunsaturated fatty acid esters, such as linoleic or linolenic acid, and possibly energy transfer to fluorescent species contained in edible oils. A weak CL emission is observed in commercial Greek EVOOs (Knossos, Spitiko, Ananias, Altis, Minerva, and Xenia) and in refined plant oils such as sunflower oils (Marata, Sanola, Sun, Mana, Sol, and Minerva) as well as in corn oils (Flora, Minerva, Marata Sun, and Sol) with potassium superoxide in the aprotic solvent dimethoxyethylene. On measuring the CL of mixtures of EVOOs with the cheaper refined oils, calibrations were produced, which can be used for the determination of the adulteration of olive oils with plant oils down to 3%. Furthermore, depending on the kind of oils, "low"-authenticity CL factors for olive oils (0.8–2.15 μmol L^{-1} gallic acid) and "high"-authenticity CL actors for seed oils (4.5–11.2 μmol L^{-1} gallic acid) were calculated.

Finally, it is necessary to mention that the most important requirement for consumers is to have the highest quality in all purchased goods. This requisite is even more obligatory when the products have health implications. Adulteration of EVOO with other vegetable oils has negative effects on oil quality and nutrition value. In fact, the adulterations are often made with refined oils impacting EVOO fatty acid composition, and contents of antioxidants and vitamins (Darmon *et al.*, 2006). In comparison to seed oils, EVOO has low levels of saturated and high levels of monounsaturated fatty acids, mainly oleic acid. Olive pomace oil is produced through extraction of olive pomace with organic solvents and has significantly lower nutritive value and price in comparison with EVOO (Kiritsakis, 1998).

29.4 Conclusion

The adulteration of olive oil is a very important issue because of its impact on quality, nutritional value, and consumer safety. Due to the inherent high cost of EVOO, the adulteration of this kind of product with cheaper or low-quality oils actually seems to be one of the most common types of fraud. The development of analytical methodologies that allow for the detection of adulterations is warranted since the detection of refined vegetable oil to EVOO at low percentages could be a very challenging task. Briefly, more efforts are needed to exploit new methods that could be assigned as reliable adulteration markers, able to detect with high selectivity, sensitivity, and accuracy blends of EVOO with other plant oils.

To prevent oil adulteration, antifraud controls require that specific tests be performed to assess the quality of oils; such controls usually rely on highly sophisticated and expensive methods (i.e., gas chromatography, liquid chromatography, Fourier transform infrared, and nuclear magnetic resonance) for monitoring the authenticity of EVOO. Moreover, the employment of several multivariate methods such as principal component analysis, canonical analysis, linear discriminant analysis, cluster analysis, partial least squares, and surface response methodology has become a prerequisite for several applications related primarily to food quality control in terms of authentication and adulteration, mainly due to a substantial simplification of the classification and grouping task.

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Advances in knowledge and technology have undoubtedly led to greater success in the fight against adulteration over the years. However, it is equally true that the same techniques and knowledge have been used by defrauders in order to invalidate the usefulness of some methods. As a result of the advances in analytical methods, or the new challenges created by fraudsters, official methods and trade standards are periodically revised and upgraded.

Over the last few years, much attention has been given to fraudulent practices associated with EVOO traceability, with special emphasis on the botanical origin due to the recent introduction in the market of high-quality monocultivar olive oil. Aiming to find traceability markers, several studies have been performed allowing the discrimination of compositional and genetical markers by exploring an alternative methodology based on the application of DNA-based detection methods in order to assess the role of DNA as a tool to detect adulteration.

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